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(54) Process for Obtaining Isoflavones

(57) Two particular isoflavones are extracted from ground-up soya beans: genistin malonate and daidzin malonate.

Translation from French

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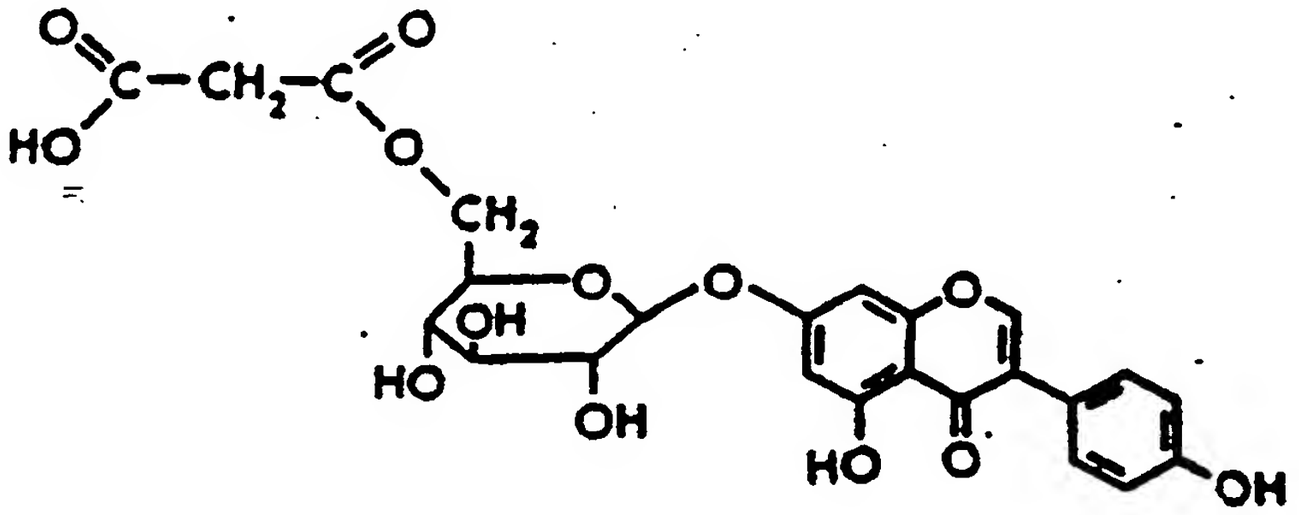
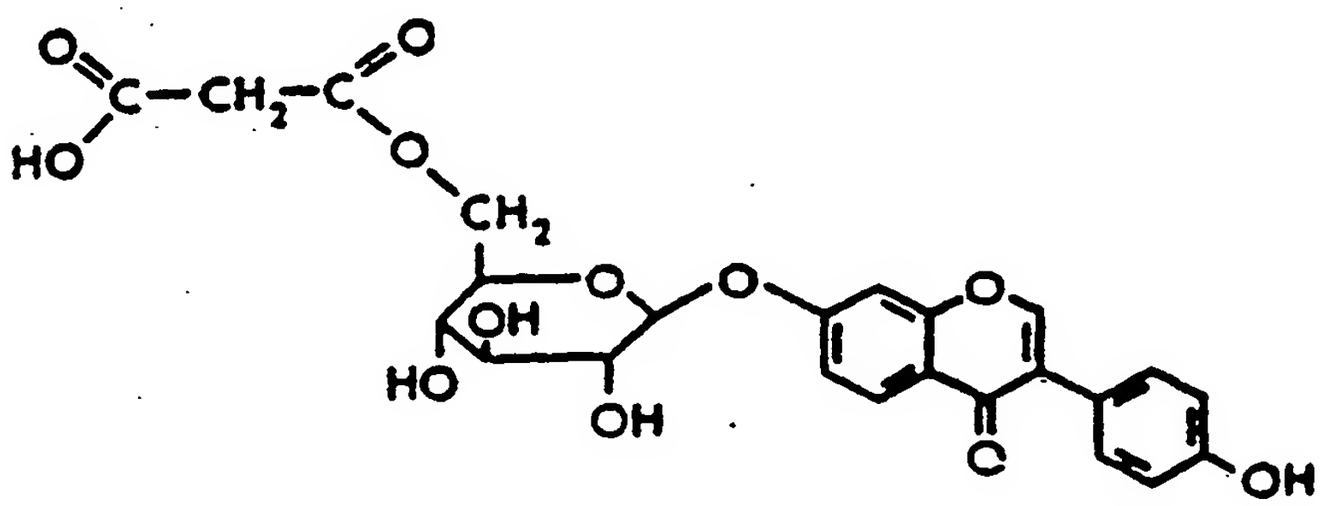
PROCESS FOR OBTAINING ISOFLAVONES

This invention relates to a process for obtaining two isoflavones, genistin malonate and daidzin malonate, and to the use of these compounds.

The article by C. M. Francis (J. Sci. Fd Agric. (1973) 24 1235) mentions the presence in clover leaves of genistin malonate, which corresponds to the formula:



It is also known from Japanese Patent No. 1146894 that daidzin malonate with the following formula:



can be extracted from the roots or stems of *Pueraria lobata*.

The object of the present invention is to provide a process for the production of these two isoflavones from an edible raw material, namely soya beans, in a simple and economic way, and with a good yield.

The process according to the invention is characterized in that ground-up soya beans are extracted with an alcohol, the crude extract obtained is buffered with an aqueous buffer solution to a pH value of 6-9, the buffer solution containing the crude extract is extracted with a water-immiscible organic solvent, the aqueous phase is recovered and acidified to pH 2.5-4, the acidified aqueous phase is extracted with a water-immiscible

organic solvent, the organic phase is recovered and neutralized to pH 6.8-7.2 and the residual compounds are separated.

With the process according to the invention, ground-up soya beans preferably having an average particle diameter of 0.5 to 0.8 mm and a maximum diameter of 1.0 to 1.1 mm can be prepared.

Before extraction, the ground-up soya beans can be defatted to remove from the soya the fats which could subsequently interfere with the correct operation of the process. Defatting may be carried out, for example, by refluxing the ground-up soya beans in an organic solvent, such as hexane, or petroleum ether. In one particular form of implementation of the invention, a mixture containing 1 part by weight ground-up soya beans to 4-6 parts by weight n-hexane may be heated with stirring (70-90 rpm) for 50-70 minutes at a temperature of 60-70°C, after which the mixture is left to cool to room temperature, the ground material is filtered and then washed with n-hexane, and the defatted ground material is dried under nitrogen for 10 to 15 hours.

The optionally defatted ground material is then extracted with an alcohol, such as methanol or ethanol for example.

This may be done, for example, by refluxing a mixture containing 1 kg defatted ground material and 6 to 8 liters of a 70-90% aqueous methanol solution with stirring (70-90 r.p.m.) for 50-100 minutes at a temperature of 70°-90°C, hot-filtering the mixture and drying the filtrate, for example under reduced pressure in a rotary evaporator at 25°-50°C. A crude extract in the form of a brown-coloured solid can be obtained in this way.

The crude extract is then buffered to bring its pH to a value of 6-9. To this end, the crude extract may be suspended or dissolved in a weak base solution, such as an aqueous $NaCO_3$ solution. This enables the subsequent extraction to be improved, the genistin and the daidzin passing into the organic phase and the desired malonates remaining in the aqueous phase. The buffer solution containing the crude extract is then extracted with a water-immiscible organic solvent, such as butanol or ethyl acetate for example. After extraction, it will be found that the organic phase mainly contains isoflavones such as genistin and daidzin, and other constituents, while the aqueous phase contains the desired compounds, and impurities.

The aqueous phase is acidified to a pH of the order of 2.5-4, for example by addition of concentrated, more particularly 5-10 N, hydrochloric acid. The acidified aqueous

phase is extracted with a water-immiscible organic solvent. It is preferable to use a solvent identical to that used for extracting the aqueous suspension or solution containing the crude extract, i.e. butanol or ethyl acetate, so that there is no need to use several different solvents, which is always dangerous. The organic phase obtained may then be neutralized by adjusting its pH to a value of 6.8-7.2, for example by addition of soda, then dried, for example by evaporation of the solvent under reduced pressure in a rotary evaporator at 25°-50°C. An impure extract containing the desired compounds is thus obtained in the form of a brown-coloured solid.

The residual compounds may then be separated from this extract, for example by performing adsorption and filtration chromatography on the impure extract, which also enables any impurities, such as the genistin and/or the daidzin still present, to be removed from the extract. This chromatography may be carried out, for example, in a column containing a gel, using an alcohol such as ethanol or a methanol/water mixture as an eluent. Several fractions containing impurities and several fractions containing a mixture of the desired compounds can be obtained. The fractions of interest may then be subjected to a second chromatography to separate the two desired compounds. To this end, reverse-phase chromatography may be carried out using, for example, a polar column containing in particular octadecyl silane groups attached to silica and an eluent or a gradient of eluents of decreasing polarity, such as for example a 10-25% solution of methanol, ethanol or acetonitrile. Two fractions each containing a different compound are obtained in this way. These fractions may be dried, in a dry evaporator for example, under reduced pressure at a temperature of 25°C to 50°C.

Two compounds are thus obtained in the form of amorphous solids, one pale yellow in colour and the other white in colour, both of which are soluble in polar solvents, such as water, methanol or ethanol.

These two compounds have been found to exhibit remarkable antioxidant properties, being capable of protecting fats, vitamins and/or oligoelements present in cosmetic or food products against oxidation.

The present invention is illustrated in more detail by the following preparation-example, identification tests for the compounds, and activity tests.

EXAMPLE OF PREPARATION OF THE COMPOUNDS

i) Defatting

5 kg n-hexane are added to 1 kg ground-up soya beans having an average particle diameter of 0.8 mm. The mixture is refluxed at a temperature of 65°C for 1 hour with continuous stirring at 80 r.p.m. This mixture is then left to cool to ambient temperature and then filtered, the ground material being recovered and dried under nitrogen for 12 hours. The dry ground material is then mixed with another 5 kg n-hexane and refluxed at 65°C for 1 hour with continuous stirring. After cooling and filtration, the resulting ground material is washed with 1 liter n-hexane. The ground material is dried under nitrogen for 10 hours. 0.7 kg of defatted ground-up soya beans are thus obtained.

ii) Preparation of the Crude Extract

A mixture containing 150 g defatted ground-up soya beans, as prepared in step i), and 1 liter of an 80% aqueous methanol solution, is prepared. The mixture is refluxed at a temperature of 80°C for 1 hour with continuous stirring at 80 r.p.m. The mixture is then hot-filtered and the filtrate is retained. The ground material is then heated with stirring for another hour at 80°C with 1 liter of 80% methanol. The mixture is hot-filtered and the filtrate obtained is added to the preceding filtrate. The combined filtrate is evaporated to dryness under reduced pressure in a rotary evaporator at a temperature of 40°C. 32.6 g crude extract in the form of a brown-coloured solid are obtained.

iii) Preparation of the Impure Extract

32.6 g of the crude extract obtained in step ii) are mixed with 150 ml of a 1 M aqueous $NaHCO_3$ solution. 150 ml n-butanol are added, the two phases are mixed and the aqueous phase is recovered by extraction, the butanol phase mainly containing daidzin and genistin. The aqueous phase is acidified to pH 3 by addition of 6.5 N hydrochloric acid. 150 ml n-butanol are added to the aqueous phase and, after mixing, a first organic phase is recovered. Another 150 ml n-butanol are added to the aqueous phase and, after mixing, a second organic phase is recovered.

The pH of the two organic phases is adjusted to 7 by addition of 1 N soda and the two phases are dried under reduced pressure in a rotary evaporator at 40°C. Impure extract in the form of a brown-coloured solid is obtained in respective yields of 2.4 g for the first phase and 1.2 g for the second phase.

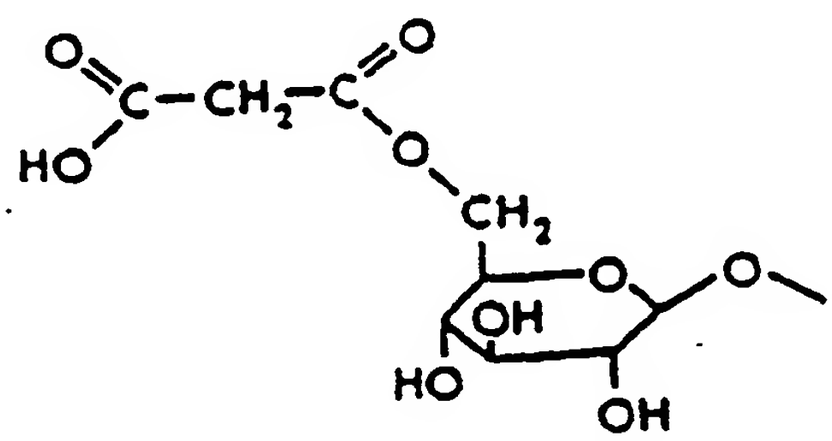
iv) Separation of the Compounds

A chromatography column 3 cm in diameter and 50 cm tall, containing a gel (Sephadex LH-20), is prepared. 2.4 g of the impure extract obtained in step iii), diluted with ethanol, is introduced into the column. The impure extract is eluted with ethanol at a rate of 2 ml per minute. A total of 17 fractions are obtained, 6 of these fractions containing the malonates of genistin and daidzin. These 6 fractions are subjected to a second chromatography in a column containing octadecyl silane groups attached to silica (Lobar RP-18). A 10% aqueous ethanol solution is used as the eluent at a rate of 2 ml per minute. Two fractions each containing a different product are finally obtained. These fractions are dried under reduced pressure in an evaporator at a temperature of 40°C. 25 mg daidzin malonate and 80 mg genistin malonate, both highly pure (92-98%), are obtained in the form of amorphous solids, the first white in colour and the second pale yellow in colour.

IDENTIFICATION TESTS ON THE GENISTIN MALONATE

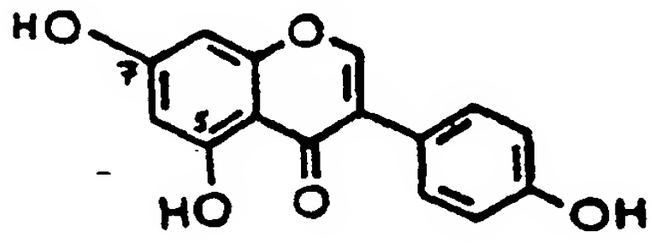
i) Position of the Sugar Group

The position of the sugar group having the formula

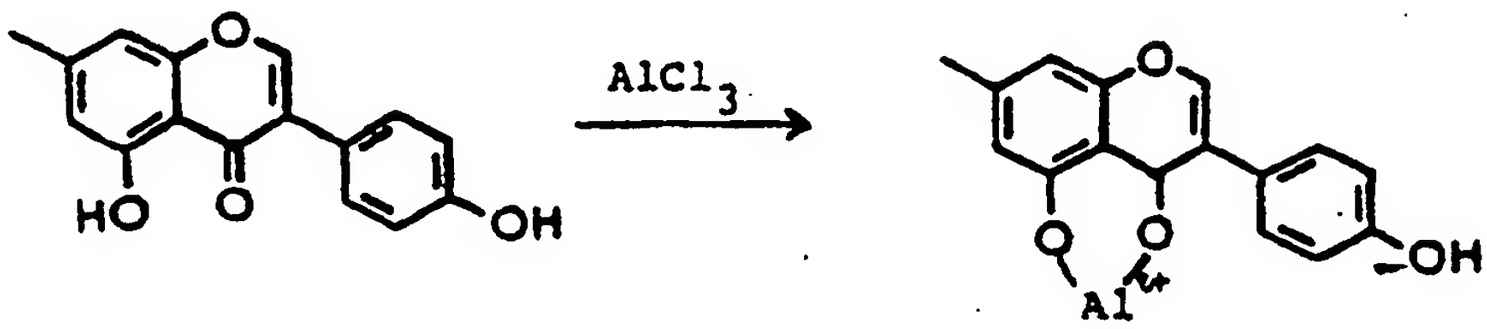


in the genistin malonate molecule is determined by a "shift reaction" with aluminium chloride.

The free OH group can be in the 5 position or the 7 position, as shown in the following formula, the other position being occupied by the above-mentioned sugar group:



If the OH group is in the 5 position, a complex will be formed in the presence of aluminium chloride in accordance with the following reaction:



Now, the λmax of the complex thus formed is about 10 to 14 nm higher than the λmax of the genistin malonate. The λmax of the genistin malonate is measured in methanol optionally in the presence of aluminium chloride. The following results are obtained:

without $AlCl_3$ λmax = 260.1 nm
with $AlCl_3$ λmax = 270.7 nm

A complex has thus been formed in the presence of aluminium chloride; the free OH group is thus in the 5 position and the sugar group in the 7 position.

ii) Decomposition in an Alkaline Medium

The retention time of malonic acid and genistin malonate, as such or in the presence of sodium hydroxide, is measured by high-performance liquid chromatography (HPLC) at 228 nm. The following results are obtained:

Malonic acid

One peak is obtained at 2.19 min.

Malonic acid + NaOH

Two peaks are obtained: one at 2.19 min corresponding to malonic acid and one at 5.03 min corresponding to sodium malonate.

Genistin malonate

One peak is obtained at 15.17 min.

Genistin malonate + NaOH

Two peaks are obtained: one at 12.90 min corresponding to genistin and one at 5.07 min corresponding to sodium malonate.

The genistin malonate is thus decomposed in the presence of sodium hydroxide into sodium malonate and genistin.

iii) Infra-Red Spectrum

The IR spectrum of genistin malonate in the solid state at a concentration of 1% in KBr shows a characteristic band at 1729.2 cm^{-1} corresponding to the carbonyl grouping of the ester (theoretical value 1735 ± 10 cm^{-1}).

iv) Mass Spectrum

The mass spectrum was prepared by two methods giving a result through deficiency ("negative FAB") and a result through excess ("positive FAB").

The following results are obtained.

A: molecular peak of the compound (genistin malonate)

B: peak corresponding to the glucoside of the isoflavone (genistin)

C. peak corresponding to the fundamental aglycone (genistein)

Genistin Malonate (Molecular Weight, g)			
	Negative FAB	Positive FAB	Theoretical value
A	517	519	518
B	431	433	432
C	269	271	270

v) NMR Spectrum

The ^{13}C nuclear magnetic resonance spectrum (NMR) of genistin malonate in dimethyl sulfoxide (DMSO-d6) at 20°C shows the following characteristic signals:

Signal	Multiplicity
45.4 ppm	CH_2
63.1 ppm	CH_2
69.7 ppm	CH
73.0 ppm	CH
74.0 ppm	CH
76.0 ppm	CH
94.3 ppm	CH
99.4 ppm	CH
99.7 ppm	CH
106.3 ppm	C
115.0 ppm	CH (2 carbons)
120.8 ppm	C
122.6 ppm	C
130.0 ppm	CH (2 carbons)
154.0 ppm	CH
157.2 ppm	C
157.7 ppm	C
161.7 ppm	C
162.5 ppm	C
168.4 ppm	C
169.2 ppm	C
179.9 ppm	C

Thus, a total of 24 carbons was found, of which 3 are carbonyl carbons (carboxylic acid and conjugated ketone), 14 are aromatic or olefinic (7 CH, 7 quaternary), 5 of the 14

being O-substituted, 1 is an anomeric CH-OH, 4 are O-substituted aliphatic CH, 1 is an O-substituted aliphatic CH_2 and 1 is a non-O-substituted aliphatic CH_2. 16 non-exchangeable protons were found as well.

DAIDZIN MALONATE IDENTIFICATION TESTS

i) Decomposition in Alkaline Medium

The retention time for malonic acid and daidzin malonate, as such or in the presence of sodium hydroxide, is measured by high-performance liquid chromatography (HPLC) at 228 nm. The following results are obtained:

Malonic acid

One peak is obtained at 2.19 min

Malonic acid + NaOH

Two peaks are obtained: one at 2.19 min corresponding to malonic acid and one at 5.08 min corresponding to sodium malonate.

Daidzin + NaOH

One peak is obtained at 9.66 min

Daidzin malonate

One peak is obtained at 13.89 min

Daidzin malonate + NaOH

The following are obtained:

a) immediately after the mixing: one peak at 11.47 min corresponding to daidzin and one peak at 8.65 min corresponding to the one obtained for daidzin + NAOH

b) 1 hour after mixing, one peak at 9.65 min corresponding to the one obtained for daidzin + NaOH

ii) Infra-Red Spectrum

The IR spectrum of daidzin malonate in the solid state at a concentration of 1% in KBr shows a characteristic band at 1734.0 cm^{-1} corresponding to the carbonyl grouping of the ester. (Theoretical value 1735 ± 10 cm^{-1}).

iii) Mass Spectrum

A mass spectrum was obtained by two methods, giving a result through deficiency (negative FAB) and a result through excess (positive FAB). The following results are obtained:

A : molecular peak of the compound (daidzin malonate)

B : peak corresponding to glucoside of the isoflavone (daidzin)

C : peak corresponding to the fundamental aglycone (daidzein)

Daidzin Malonate (Molecular Weight, g)

Genistin Malonate (Molecular Weight, g)			
	Negative FAB	Positive FAB	Theoretical value
A	501	503	502
B	415	417	416
C	253	255	254

iv) NMR Spectrum

The ^{13}C nuclear magnetic resonance spectrum (NMR) of daidzin malonate in dimethyl sulfoxide (DMSO-d6) at 20°C shows the following characteristic signals:

Signal	Multiplicity
45.2 ppm	CH_2
63.0 ppm	CH_2
69.7 ppm	CH
73.0 ppm	CH
74.0 ppm	CH
76.1 ppm	CH
99.7 ppm	CH
103.4 ppm	CH
114.9 ppm	CH
115.3 ppm	C
118.4 ppm	CH (2 carbons)
122.0 ppm	C
123.6 ppm	C
126.9 ppm	CH (2 carbons)
129.9 ppm	CH
153.2 ppm	C
156.8 ppm	C
157.4 ppm	C
161.0 ppm	C
168.3 ppm	C
169.1 ppm	C
174.7 ppm	C

Thus, a total of 24 carbons was found, of which 3 are carbonyl carbons (carboxylic acid and conjugated ketone), 14 are aromatic or olefinic (8 CH, 6 quaternary) 4 of the 14

being O-substituted, 1 is an anomeric carbon (CH-OH), 4 are O-substituted aliphatic CH, 1 is an O-substituted aliphatic CH_2 and 1 is a non O-substituted aliphatic CH_2. 17 non-exchangeable protons were found as well.

ACTIVITY TESTS FOR THE COMPOUNDS

i) Qualitative Analysis

It is known that β-carotene is oxidized in the presence of linoleic acid by ultraviolet (UV) irradiation. The oxidation is shown by the discoloration of the β-carotene.

One drop of an aqueous solution of daidzin, genistin, daidzin malonate or genistin malonate is applied to a support plate for thin-layer chromatography. A solution containing 100 mg β-carotene and 1 ml linoleic acid in 100 ml chloroform is then vaporized on the plate. The plate turns yellow. The plate is placed beneath a UV light source and left there until discoloration occurs. It can be seen that, at the places where the genistin and the daidzin were applied, the plate is not discoloured and is still yellow in colour. This is due to the fact that the daidzin and the genistin have antioxidant properties which prevent oxidation and, hence, discoloration of the β-carotene. It can also be seen that the plate has the same yellow colour at the places where the malonates of genistin and daidzin were applied. Accordingly, these two compounds also exhibit antioxidant properties.

ii) Quantitative Analysis by Spectrophotometry

0.01 M solutions, in methanol, of daidzein, genistein, daidzin, genistin, daidzin malonate and genistin malonate are prepared.

Several solutions of gallic acid in methanol varying in concentration from 0.2 to 20 gl^{-1} are prepared in the same way, as standards.

A solution containing 100 mg β-carotene, 1 ml linoleic acid and 100 ml chloroform is also prepared as a reactant.

First samples containing 20 µl of the solution to be measured, 60 µl reactant and 2 ml methanol are prepared, and second samples, as controls, containing 20 ml of the solution to be measured and 2 ml methanol, are also prepared.

The absorption of the sample in relation to its control sample is measured at 450 nm, just after preparation, after which the two samples are exposed to ultraviolet radiation for 12 minutes and their absorption is remeasured.

The results obtained for the isoflavones can then be compared with the results obtained for the various solutions of gallic acid, enabling the following equivalences to be determined:

0.010 M Isoflavone Solution	Molar concentration of the gallic acid solution having the same antioxidant power
Daidzein	0.020 M
Daidzin	0.022 M
Daidzin malonate	0.024 M
Genistein	0.028 M
Genistin	0.027 M
Genistin malonate	0.029 M

It can be seen that the 0.010 M solutions of daidzin malonate or genistin malonate have the same effect as the 0.010 M solutions of daidzin or genistin and the 0.024 M and 0.029 M solutions of gallic acid.

iii) Oxidation Test

The Rancimat accelerated oxidation test is used to determine the induction time for chicken fat stabilized by addition of genistin malonate or daidzin malonate.

The Rancimat® test consists in passing air through a reaction tube containing a sample of 5 g fat at 100°C and measuring the conductivity of the volatile secondary products formed during oxidation and entrained with the stream of air. The induction time is graphically determined from the recorded conductivity/time curve by intersection of the tangent to the curve with the time axis.

The following results are obtained for chicken fat containing 500 ppm genistin malonate or daidzin malonate or none at all (control).

	Induction time (h)
Control	6.96
Genistin malonate 500 ppm	8.10
Daidzin malonate 500 ppm	7.68

The antioxidant effect of genistin malonate and daidzin malonate is thus clearly confirmed.

What we claim is:

1. A process for obtaining isoflavones, characterized in that ground-up soya beans are extracted with alcohol, the crude extract obtained is buffered with an aqueous buffer solution to achieve a pH of 6–9, the buffer solution containing the crude extract is extracted with a non-water-mixable organic solvent, the aqueous phase is recovered and acidified to pH 2.5–4, the acidified aqueous phase is extracted with a non-water-mixable organic solvent, the organic phase is recovered and neutralized to pH 6.8–7.2, and the residual compounds are separated.

2. A process as claimed in claim 1, characterized in that the ground up soya-beans are extracted with an aqueous 70-90% methanol solution, by heating to 70-90°C for 50-100 minutes.

3. A process as claimed in claim 1, characterized in that the non-water-mixable organic solvent is butanol.

4. A process for protecting a food product or cosmetic product from oxidation, characterized in that an amount of genistin malonate and/or daidzin malonate that will effect such protection is incorporated in the product.